Comments from Reviewer 1

1) 1st paragraph: If possible, please provide information on current approaches for FC-HCS.

We added a short description of FC-HCS approaches, and provided references with more detail explanations (references 9-10).

2) page 2, paragraph 3: it would be good to add again that there were 5 PBMC plates(?), this would help the reader understand the basic setup

Added "5 replicate" plates to pg2, paragraph 2.

3) There's a typo on page 2, paragraph 3: equivalence of

Fixed

4) Materials and Methods section:
Flow Cytometry Data:
For the readers convenience I would suggest providing the full link where the FCM files can be downloaded and the name of the file.

Fixed

5) plateCore and FlowJo section:
Maybe these two sections could be structured in the same way to make it clearer which steps are corresponding and where these two approaches differ.
PlateCore section:
In the plateCore section it is a bit difficult to figure out where the processes described in the first paragraph fit into the processes described in the second paragraph. Also, is there a step in plateCore that corresponds to the assigning of the groups in FlowJo?

The plateCore and FlowJo descriptions have been revised in a list format to make it easier to identify the corresponding steps.

6) The label of figure 1 states that plateCore makes it easier to aggregate multiple plates into an experiment level object. Where does this step fit in the workflow? This seems to be an advancement in comparison with other software, so it would be worthwhile to mention it in the main text as well.

The function used to aggregate the plates, named fpBind, is now described in the plateCore section.

7) FlowJo paragraph: Was the assigning to the 30 groups a random?

Group assignments were based on isotype and fluorophore availability. New versions of FACS CAP have biological reasons behind the antibody layout. A short description of the logic behind isotype group and antibody combinations in the wells was added to the plateCore section.

8) Results:
It would have been interesting to know how the results from the original analysis compare to those that you have. Was the analysis done not comparable / not available?

Unfortunately, we are restricted to discussing a limited set of markers, as we are not authorized to share detailed results from the original analysis. Results from the original FlowJo analysis for the percentage of positive cells are masked are provided, although the antibody names have been redacted.

9) FlowJo Output:
The authors say that markers that have been previously characterize using BD FACS CAP with >= 90% of the cells above the treshold are usually confirmed as positive and <= 10% often the result of non-specific binding. Could you clarify if you are refering to the before-mentioned single color titration and competition experiments here, or how was this confirmation done?

Yes, the conformation was performed using single color titration and competition experiments. The sentence has been changed to include this information.

10) plateCore versus FlowJo:
first paragraph: 'Isotype controls are used to determine the threshold between background staining and specific binding of an antibody conjugate to its target.' I think this should already be explained in the Materials and Methods section, though it might be repeated here.

This sentence is redundant since isotype controls are explained in the Materials and Methods section, however we believe it is helpful to Advances in Bioinformatics readers who are not familiar with FCM.

11) Page 5, first paragraph: “as evident from the density plots”: it would be a bit easier for the reader if you could add the corresponding number of the figures.

Added references to figures 5 and 8 in the text

12) Page 5, Third paragraph:
In the abstract you claim that results for flowJo and plateCore are in good agreement. It would be interesting to have at least a rough quantification how often there was considerable disagreement between the two methods. It's not so

easy to see this in the figures as there are a lot clearly positive and clearly negative markers and these points are overlapping very much in the figure.

We added r-square values to the Figure 3 that provide a rough idea of how well the methods agree.

13) Discussion:
The authors claim that they realized individual isotype gates should not be changed by cytometrists to avoid bias. However, in the results section (page 5, last section before quality assessment) the authors wrote that more focused studies would need to be performed to determine whether, the cytometrists were correct in this case or whether they just add noise. Therefore I think the authors should either be more cautious in their advise or justify their realization here (eg why is their gating quality assessment more objective?).

In the discussion we suggest that cytometrists should not adjust individual gates in FC-HCS experiments, rather that these types of changes should be made on an experiment-wide basis. Since individual gating changes are often based on prior knowledge about the antibody-dye conjugate (i.e. the fluorophore is bright or the antibody is sticky) and not on information from the experiment, such changes typically add to the overall noise level of the measured results and make it difficult to compare results across multiple samples. Regardless of whether or not additional studies focused on CD112 confirm that it is expressed, changing the gate based on presumed negative staining in a related test sample (CD109 IgG1-PE) is subjective and not reproducible. Also, the quality assessment we propose in this paper is more objective since they are based on the results from the analysis of the experiment in question, and not on prior knowledge about how a particular antibody conjugate performs.

14) Figure 2: The axis annotation is a bit unusual. This is a minor `beauty' fault, but would be nice if it could be fixed.

Fixed

15) References:
There are a number of references that should be checked / corrected:
The author names should be used in the format suggested by the journal. [4]: I found this source quoted with a slightly different title: flowcore: a Bioconcuctor package for high throughput cytometry

Fixed

16) [5]: I found this source quoted with a slightly different title: Data quality assessment of ungated flow cytometry data in high throughput experiments [8]: page 878-879

Fixed

Comments from Reviewer 2

1) Why was a 2 standard residual threshold chosen?

Any particular threshold for classifying data points as outliers is ultimately arbitrary. We chose 2 standardized residuals in a conservative attempt to ensure that any questionable automated gating decisions were examined in detail. We anticipate developing more sophisticated approaches to outlier detection as we analyze additional cell types, and also by incorporating information about multiple cell populations.

2) How does the prescribed gating procedure perform in comparison to an inconsistent e.g. random or biased gating, when evaluated using the methods described by the authors?

One of the main goals in the automated analysis was to replicate the results from manual gating in FlowJo, so our evaluation focuses on comparing plateCore and FlowJo output. Although it would be possible to compare plateCore output to random gates and static gating strategies, we do not believe it would useful to include these results in this paper. FlowJo and plateCore analysis are an obvious improvement over randomly selecting a level in the 10-bit range of channels on the FACSCalibur. We have also evaluated static gates and while it is often possible to find a setting that works for a specific antibody-dye conjugate on replicate plates, changes in instrument settings, variation in non-specific binding, and variation in the intensity of the fluorophores make it difficult to identify a single fixed value for all antibody-dye conjugates across multiple donors in an experiment.

3) Figure 1 compares a manual and the plateCore analysis work flow, but the last step given is the generation of summary statistics. The Authors should include the steps necessary for generating the most important plots such as Figure 7.

The code used to generate figure 7 has been included in the supplementary material.

4) Figure 2,3,4 and 7 suffer from "overplotting" due to large symbols. The use of smaller symbols could enhance readability.

We decreased the size of the symbols by in Figures 2-4. Symbols in Figure 7 are already small relative to the size of the panel and become difficult to distinguish if they are smaller.

5) Figure 2-4 could be improved plotting the mean percentage versus the difference, as most bioinformatics practitioners are familiar with M-A plots.

We believe that bivariate scatterplots in Figures 2-4 allow readers to quickly and easily interpret the percentage results. Ideally we would like to compare the actual gate settings from FlowJo and plateCore, rather than the proportion of positive cells, but unfortunately the FlowJo workspace with gates contains proprietary information about the BD FACS™ CAP plate configuration. We agree that making M-A style plots for MFIs or isotype cutoffs (which are simply fluorescent signal thresholds) would be an improvement if we could release detailed information from the FlowJo analysis.

6) The analysis scripts referenced in the manuscript were not available for review, thus the following is based on reviewing the development version in the Bioconductor SVN and may not apply to the scripts submitted by the authors. The example scripts could not be executed without modification by the reviewer. Most errors were stemming from discrepancies between the naming of fluorescent channels in the example data set and the names used in the scripts. Furthermore, after adjusting the channel names to match the content of the example data, the plotPlate() visualization function only generated trivial output. The Authors should verify that these problems are absent in the published scripts.

Updates to plateCore have been committed to Bioconductor, which should resolve these problems.